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Docket No: 210352US0X

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF:	:	
Ryuichiro KURANE, et al.	:	
SERIAL NO: 09/891,517	:	ATTN: BOX MISSING PART
FILED: June 27, 2001	:	
FOR: NOVEL NUCLEIC ACID PROBES, METHOD FOR DETERMINING CONCENTRATIONS OF NUCLEIC ACID BY USING THE PROBES, AND METHOD FOR ANALYZING DATA OBTAINED BY THE METHOD	:	

FILING OF CERTIFIED ENGLISH TRANSLATION UNDER 37 CFR 1.52(d)

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Responsive to the Notice to File Missing Parts of Application (Form PTO-1533) dated July 17, 2001, Applicants submit herewith a certified English translation of example 2 to example 8 in the specification , as filed, in accordance with the provisions of 37 C.F.R. §1.52(d).

The required fee was paid at the time of filing of the application.

In light of the foregoing, this application is deemed to be in proper condition for examination and such favorable action is earnestly solicited.

Respectfully submitted,

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DECLARATION

I, Tadashi Tsukamoto of 41-8, Utsukushigaoka 3-chome, Aoba-ku, Yokohama, Kanagawa 225-0002, Japan do solemnly and sincerely declare that I well understand both Japanese and English languages.

The translation attached hereto is a true and accurate translation of pages 109 through 118 (Examples 2 through 8) of U.S. patent application Serial No. 09/891,517 which were filed in Japanese.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

This 8th day of September, 2001

Tadashi Tsukamoto

Example

Synthesis of target nucleic acid

An oligonucleotide the base sequence of which was (5')GGGGGGAAAAAAAAA(3') was synthesized in a similar manner as in the synthesis of the above-described oligonucleotide, and provided as a target nucleic acid to which the present invention is applicable.

Example 3

Measurement of the intensity of fluorescence from a reaction system in which a probe according to the present invention had been hybridized with the target nucleic acid

Buffer (2 M NaCl, 200 mM Tris-HCl; pH 7.2)(500 µL) was added to a quartz cell (10 mm x 10 mm)(capacity: 4 mL), followed by the addition of sterilized distilled water (1460 µL). The resulting mixture was then stirred. While maintaining the mixture at 35℃, the intensity of fluorescence was measured in the course of time [exciting wavelength: 581 nm (8 nm wide); wavelength of measuring fluorescence: 603 nm (8 nm wide)]. A target nucleic acid solution the concentration of which was 160 nM (32.0 µL) was then added, followed by stirring. The intensity of fluorescence was measured in the course of time under the same conditions as described above. The results are diagrammatically shown in FIG. 1. It is understood from the diagram that the addition of a target nucleic acid leads to an increase in the intensity of fluorescence and this increment

is leveled off in an extremely short time, specifically in 100 seconds (1 minute and 40 seconds) [incidentally, about 15 minutes are required in the case of a molecule beacon: Nature Biotechnology, **14**, 303-308 (1998)]. This indicates that the method of the present invention for the measurement of a nucleic acid can be performed in a short time.

Example 4

Measurement of the target nucleic acid

Under similar conditions as described above except that the concentration of the target nucleic acid were varied in various ways, the intensity of fluorescence was measured at the varied concentrations. The results are diagrammatically illustrated in FIG. 2. It has been found from the diagram that the intensity of fluorescence increases with the concentration of a target nucleic acid and their relationship is proportional.

From the foregoing results, it has been confirmed that the use of a nucleic acid probe according to the present invention permits measurement of a nucleic acid with good accuracy.

Example 5

(Effect of the distance between a fluorescent dye and a quencher substance)

A deoxyribooligonucleotide the base sequence of which is shown in FIG. 3 was synthesized as in Example 1. In a similar manner as in Example 1, a probe was prepared by labeling a

phosphate group at the 5'end of the deoxyribooligonucleotide with a fluorescent dye, Texas Red, and also by labeling an OH group on the C atom at the 6-position of the thymine base with a quencher substance, Dabcyl. The labeled thymine base was then shifted one base by one base toward the 3'end. In this manner, twenty (20) probes according to the present invention were synthesized. To those probes, complementary target deoxyribooligonucleotides were hybridized, respectively. Changes in the intensity of fluorescence through the hybridization were measured.

Tris buffer (2 M NaCl, 200 mM Tris-HCl; pH 7.2)(500 µL) was added to a quartz cell (the same cell as that used in Example 3), followed by the addition of sterilized distilled water (1460 µL). The resulting mixture was then stirred. A 10 µM solution of the probe according to the present invention (8.0 µL) was added to the mixture, followed by stirring (final concentration of the probe: 40 nM). While maintaining the mixture at 35℃, fluorescence was measured [exciting wavelength: 581 nm; wavelength of measuring fluorescence: 603 nm; slit width: 8 nm (both)]. A 10 µM solution of the target deoxyribooligonucleotide (32.0 µL) was then added, followed by stirring (final concentration of the target deoxyribooligonucleotide: 160 nM). Measurement of fluorescence was thereafter conducted in the course of time under the same conditions as described above.

The results are diagrammatically shown in FIG. 4. As is

appreciated readily from the diagram, it was observed that in most of the fluorescence emitting probes of the present invention dually modified by Dabcyl and Texas Red, hybridization with the target deoxyribooligonucleotide leads to an increase in the emission of fluorescence compared with the emission of fluorescence before the hybridization. Further, maximum emission of fluorescence was observed when the inter-base distance from the base having the phosphate group labeled with Texas Red to the Dabcyl-labeled base (when counted by assuming that the base number of the base labeled with Text Red was the 0^{th} base) was 6 bases long. The emission of fluorescence at that time was about 11 times. When the inter-base distance was 16 bases long, large emission of fluorescence was also observed. The emission of fluorescence at that time was about 11 times as in the case of the 6 bases. As a DNA helix makes a turn with 10 bases, the 6^{th} and 16^{th} bases as observed from the 5'end base are located substantially on the opposite side of the helix. It is therefore considered that, when the 6^{th} and 16^{th} bases were labeled with the quencher substance, quenching of fluorescence took place based on transfer of electrons between Dabcyl and Texas Red when the deoxyribooligonucleotide is in the single-stranded form, but as a result of physical separation of Dabcyl and Texas Red from each other by the hybridization, the quenching of fluorescence based on the transfer of electrons was canceled and Texas Red

emitted fluorescence.

Example 6

(Relationship between fluorescent dye and intensity of emitted fluorescence)

An investigation was conducted about the kinds of fluorescent dyes in fluorescent emitting probes according to the present invention. An experiment was carried out in a similar manner as in Example 5 except that the inter-base distance between each fluorescent dye and Dabcyl was set at 6 bases long and the width of a slit for fluorescence measurement was set at 5 nm in both excitation and measurement. The results are presented in Table 1.

An absorption of Dabcyl as a quencher appears at 400 to 500 nm. Many of probes with large emission of fluorescence, however, emitted fluorescence at wavelengths substantially shifted from the absorption of Dabcyl, that is, at wavelengths longer than 550 nm. In the case of a fluorescent dye which emits fluorescence at wavelengths longer than 550 nm, the mechanism of fluorescence quenching by Dabcyl is considered to be attributable primarily to transfer of photoexited electrons rather than FRET. Since Dabcyl and the fluorescent dye are physically separated from each other as a result of a change in the stereostructure of the probe, the fluorescence quenching phenomenon by the transfer of photoexcited electrons is cancelled. In the case of a fluorescent dye such as FITC which

emits fluorescence at wavelengths close to the absorption of Dabcyl, it is considered that, even when Dabcyl and a fluorescent dye are physically separated as a result of a change in the stereostructure and the fluorescence quenching phenomenon based on transfer of photoexcited electrons is hence cancelled, no substantial emission of fluorescence takes place from quenching of fluorescence by FRET because the quenching of fluorescence by FRET prevails. Accordingly, a dye capable of satisfying the following three conditions is desired as a fluorescent dye for use in a fluorescence emitting probe according to the present invention: (1) the fluorescence quenching phenomenon based on transfer of photoexcited electrons occurs between the fluorescent dye and Dabcyl; (2) Fluorescence of wavelengths substantially shifted from the absorption of Dabcyl is emitted; and (3) a strong interaction exists between the fluorescent dye and Dabcyl to reduce the intensity of fluorescence before hybridization (in other words, to further facilitate the occurrence of the fluorescence quenching phenomenon by transfer of photoexcited electrons).

Table 1

Relationship between Various Fluorescent Dyes and Intensity of Emitted Fluorescence

Fluorescent dye	Exciting wavelength	Wavelength of fluorescence	Intensity of fluorescence before addition of target nucleic acid (A)	Intensity of fluorescence after addition of target nucleic acid (B)	A/B
FITC	480	510	11.5	18.5	1.6
TET	500	530	36.8	48.0	1.3
HEX	520	540	3.9	7.4	1.9
Cy3	540	560	2.0	7.4	3.7
Bodipy 581/591	555	582	1.6	6.4	4.0
Alexa531	500	539	7.3	26.0	3.6
6-ROX	560	590	4.8	18.9	3.9
Alexa594	575	603	2.2	15.22	6.9
Bodipy TR	585	615	2.4	9.6	4.0
Texas Red	585	603	2.1	21	10

Example 7

(Probe with intra-chain bases modified by fluorescent dye and quencher)

A probe with intra-chain bases modified by a fluorescent dye and a quencher and a target deoxyribooligonucleotide, such as those shown in FIG. 5, were synthesized in a similar manner as in Example 1 with the following exceptions: (1) using "Amino-Modifier C6 dT" (product of Glen Research Corporation, VA, U.S.A) in place of "5'Amino-Modider Cy Kit" (product of Glen Research Corporation, VA, U.S.A), the probe was modified with Texas Red; (2) Dabcyl was introduced directly into the base chain by using "Dabcyl dT" (product of Glen Research Corporation, VA, U.S.A) instead of modifying the probe with Dabcyl by means of "5'Amino-Modifier C6 Kit" (product of Glen Research Corporation, VA, U.S.A); and (3) the Dabcyl-modification step and the subsequent purification step were omitted accordingly.

An investigation was then conducted in a similar manner as in Example 5 to determine whether or not the probe so obtained would be actually usable. A further investigation was also made for possible effects of the distance between the bases labeled with the quencher (Dabcyl) and the fluorescent dye (Texas Red), respectively. The results are diagrammatically illustrated in FIG. 6. As is evident from the results, it has been found that even a probe with intra-chain bases modified with a fluorescent

dye and a quencher, respectively, is actually usable. Like the probe with the 5'end phosphate group modified with Texas Red, maximum emission of fluorescence was observed when the inter-base distance between Texas Red and Dabcyl was 6 bases long or 16 bases long. The intensity of fluorescence emitted at that time was about 10 times higher compared with the fluorescence intensity before the hybridization.

Examples 8-31 and Comparative Example 1 relate to fluorescence quenching probes according to the present invention.

Example 8

Preparation of a nucleic acid probe to be hybridized to the nucleic acid base sequence 16S rRNA of *Escherichia coli*, namely, having the base sequence of (3')CCGCTCACGC ATC(5') was conducted as will be described hereinafter.

Preparation of nucleic acid probe

A deoxynucleotide, which had the base sequence of (3')CCGCTCACGC ATC(5') and contained $-(CH_2)_7-NH_2$ bonded to the OH group on the carbon atom at the 3' position of deoxyribose at the 3'end of the oligodeoxyribonucleotide, was purchased from Midland Certified Reagent Company, TX, U.S.A. From Molecular Probes, Inc., "FluoReporter Kit F-6082" (trade name) was also purchased, which contained not only "BODIPY FL" propionic acid succinimidyl ester but also a reagent for

conjugating the compound to the amine derivative of the oligonucleotide. The kit was caused to act on the above-purchased oligonucleotide, whereby a nucleic acid probe labeled with "BODIPY FL" was synthesized for use in this Example.

Purification of synthesized product

The synthesized product was dried into a dry product. The dry product was dissolved in 0.5 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.0). The solution was subjected to gel filtration through "NAP-25 Column" (trade name, product of Pharmacia AB, Uppsala, Sweden), whereby unreacted substances were removed. Further, reversed phase HPLC (B gradient: 15 to 65%, 25 minutes) was conducted under the below-described conditions. An eluted main fraction was collected. The collected fraction was lyophilized, whereby a nucleic acid probe was obtained with a yield of 23% as calculated relative to 2 mM of the starting oligonucleotide.

The above-described reversed phase chromatography was conducted under the following conditions:

Eluting solvent A: 0.05 N TEAA 5% CH_3CN

Eluting solvent B (for gradient elution): 0.05 N TEAA 40% CH_3CN

Column: "CAPCEL PAK C18" (trade name), 6 x 250 mm

Elution rate: 1.0 mL/min

Temperature: 40℃

Detection: 254 nm